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Voltage-independent Adaptation of Mechanosensitive Channels in *Escherichia coli* Protoplasts

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Abstract. Mechanosensitive (MS) ion channels, with 560 pS conductance, opened transiently by rapid application of suction pulses to patches of *E. coli* protoplast membrane. The adaptation phase of the response was voltage-independent. Application of strong suction pulses, which were sufficient to cause saturation of the MS current, did not abolish the adaptation. Multiple-pulse experimental protocols revealed that once MS channels had fully adapted, they could be reactivated by a second suction pulse of similar amplitude, providing the time between pulses was long enough and suction had been released between pulses. Limited proteolysis (0.2 mg/ml pronase applied to the cytoplasmic side of the membrane patch) reduced the number of open channels without affecting the adaptation. Exposing patches to higher levels of pronase (1 mg/ml) removed responsiveness of the channel to suction and abolished adaptation consistent with disruption of the tension transmission mechanism responsible for activating the MS channel. Based on these data we discuss a mechanism for mechanosensitivity mediated by a cytoplasmic domain of the MS channel molecule or associated protein.

Key words: Mechanosensation — Ion channel — Adaptation — *Escherichia coli* — Proteolysis

Introduction

Mechanosensitive (MS) ion channels are membrane proteins that respond to membrane stretch and may act as physiological mechanotransducers. MS channels have been found in a variety of cell types including Gram-negative bacteria (for reviews, *see* Sachs, 1992; Martinac, 1993; Sackin, 1995; Sukharev et al., 1997). The envelope of *Escherichia coli*, like other Gram-negative bacteria contains two membranes: the outer and the inner, cytoplasmic membrane. Patch clamp studies on giant *E. coli* spheroplasts (two membrane objects) have revealed two types of MS channels of 1 nS and 3 nS conductance denoted MscS (MS channel ''small'') and MscL (MS channel ''large''), respectively (Martinac et al., 1987; Sukharev et al., 1993). MscL was the first MS channel ever cloned and sequenced (Sukharev et al., 1994; Sukharev et al., 1997). MS channels have been also found in *E. coli* giant protoplasts (one-membrane objects, containing cytoplasmic membrane only) (Kubalski et al., 1992; Cui, Smith & Adler, 1995; Berrier et al., 1996) and in liposomes reconstituted from the inner membrane fraction (Delcour et al., 1989; Berrier et al., 1989).

Decline in activity of mechanoreceptors in response to sustained mechanical stimulation (adaptation) has been demonstrated on several preparations. Voltage-dependent adaptation of mechanoelectrical transduction has been shown in hair cells (for review, *see* Hudspeth & Gillespie, 1994). Yeast (Gustin et al., 1988; Zhou & Kung, 1992), *Xenopus* oocytes (Hamill & McBride, 1992) and C6 glioma cells (Bowman & Lohr, 1996) displayed voltage-dependent adaptation at the level of single MS channel activities. The MS channel adaptation, in all these cases, was fragile and moderate suctions resulted in its irreversible removal. The irreversible loss of adaptation can be attributed to the loss or disruption of cytoplasmic regulatory domains of the MS channel molecule or/and a regulatory neighboring protein. It is also possible that some cytoskeletal restraining elements were lost (Hamill & McBride, 1997). At low suctions, however, the adapted MS channels could be reactivated by application of higher suction steps indicating that adaptation represents a change in sensitivity of gating instead of channel inactivation (Hamill & McBride, 1992; 1994). Adaptation of *E. coli* MS channels has not been inves-

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tigated in detail. MscL, recorded from spheroplasts, did not show obvious adaptation (Sukharev et al., 1997) and closure of the MscS during sustained suction was demonstrated upon high pipette voltage (+90 and +140 mV in patches excised from spheroplast and from liposome respectively) (Sukharev et al., 1993). In this study we investigate the phenomenon of adaptation of the 560-pS MS channel from *E. coli* protoplasts. This MS channel may represent MscS with an altered kinetics. The MscS, recorded from spheroplasts in different ionic conditions, displayed no adaptation (Martinac et al., 1987; Martinac, Adler & Kung, 1990; Kubalski et al., 1993). Adaptation of the 560-pS MS channel was studied here in relation to the amount and duration of the applied suction, and polarity of the membrane potential. We were also interested if proteolytic treatment of the cytoplasmic side of the membrane patch would affect the adaptation and tension transmission mechanism of the MS channels.

We are aware that the term ''MS channel adaptation'' used throughout the paper simplifies to a large extent the possible mechanism that is responsible for a decline of MS channels activities. Adaptation may not be a property of the MS channel molecule itself and may reflect changes in associated proteins and/or cytoskeleton. Moreover, the MS channel protein may act as a reporter of tension in the membrane patch that may relax during maintained stimulation. The latter possibility is discussed.

Materials and Methods

Bacterial Strains

All experiments were performed on a strain Frag126, a derivative of *E. coli* K12. Frag126 (F^-, *lacZ, thi, rha, kdp*ABC5, *kef*B::Tn*10*) was kindly provided by Wolfgang Epstein.

Patch Clamp Recording and Data Analysis

Giant spheroplasts of *E. coli* were prepared as described previously (Martinac et al., 1987). Protoplasts used in patch-clamp experiments (Hamill et al., 1981) were generated by removing the outer membrane from the spheroplasts (Kubalski, 1995; Cui et al., 1995). All experiments were performed at room temperature using EPC7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). After giga-seal formation, single-channel activities were recorded from inside-out excised patches obtained by brief air exposure of the tip of the patch pipette (borosilicate glass, WPI, Sarasota, FL). All pipettes of controlled shape and diameter had a bubble number of 3.2 in 100% ethanol corresponding to resistances in recording solution of 9–11 MΩ. Bath solution was (in mM): 150 KCl, 400 sorbitol, 4 $CaCl_2$, 5 Tris-HCl, pH 7.2, whereas the pipette solution was the same except that the sorbitol concentration was 300 mM. Unitary conductances were calculated for symmetric K^+ conditions from the slopes of current-voltage relationships obtained in voltage range between −40 and +40 mV. Suction was applied pneumatically to the patch pipettes by a 10-ml syringe, together with two in-line, three-way valves and was monitored by a pressure manometer PM015D (±1.5 psi) (WPI, Sarasota, FL). 60-sec intervals or longer were maintained between applications of suction pulses that were not shorter than 20 sec. The recordings were filtered at 1 kHz with a 4-pole Bessel filter (Frequency Devices, Haverhill, MA), acquired and stored on a computer running the Digidata 1200A/Axoscope 1.0 acquisition system and analyzed with pCLAMP6 software (Axon Instruments, Foster City, CA).

The mean open probability, P_o during the pressure pulse, was calculated by integrating the current passing through all active channels during the recording time (I) (not shorter than 30 sec) and dividing this integral by the current through a single open channel (i), multiplied by the number of active channels (N) according to the formula: $P_o = I/N\,i$. The total open probability, NP_o, was calculated as: $NP_o = I/i$. The P_o data were plotted against applied suction and fitted with Boltzmann curves as described previously (Martinac et al., 1987). The Boltzmann distribution was given by:

$$P_o = \{\exp[(p - p_{1/2})/S_p]\}/\{1 + \exp[(p - p_{1/2})/S_p]\}$$

in which p is the suction, $p_{1/2}$ is the suction at which the channel is open half of the time, S_p is the slope of the plot of $ln[P_o/(1 - P_o)]$ *vs.* suction.

Data points were plotted and fitted to single exponential functions using Origin 4.0 (Microcal™ Software).

Chemicals

Cephalexin, lysozyme, DNA-ase and pronase (from *Streptomyces griseus*) were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were of reagent grade. Pronase was dissolved and stored at 20 mg/ml at −20°C. The final dilution to 0.2 or 1 mg/ml was made just before the experiment.

Results

The results presented in this study were obtained on excised inside-out membrane patches from *E. coli* protoplasts. Application of suction (negative pressure) to the recording patch pipette resulted in activation of three types of MS ion channels, which differed in size and in threshold pressure required for their activation. Their single-channel conductances were 150 pS ± 7 ($n = 4$), 564 ± 19 pS ($n = 5$) and 1067 ± 19 pS ($n = 4$) in symmetric 150 mM KCl. Activities of 150-pS and 560-pS MS ion channels could be usually seen at suctions below 200-mm Hg. The 1070-pS MS channel (representing MscL) required higher (>200-mm Hg) suctions to be activated. The 560-pS and 1070-pS channels were seen most frequently and usually both of them could be detected in the same membrane patch. Typical sequence of events when suction was applied to the membrane patch containing these two types of MS channels is shown in Fig. 1*A*. At lower suctions (130- and 168-mm Hg), activities of the 560-pS MS channels were observed (Fig. 1*A*, two upper traces). At 221-mm Hg, most of the 560-pS MS channels remained open during the entire suction pulse and in addition single openings of the 1070-pS MS channel were seen (Fig. 1*A*, third trace from the top). The two types of MS channels were kinetically

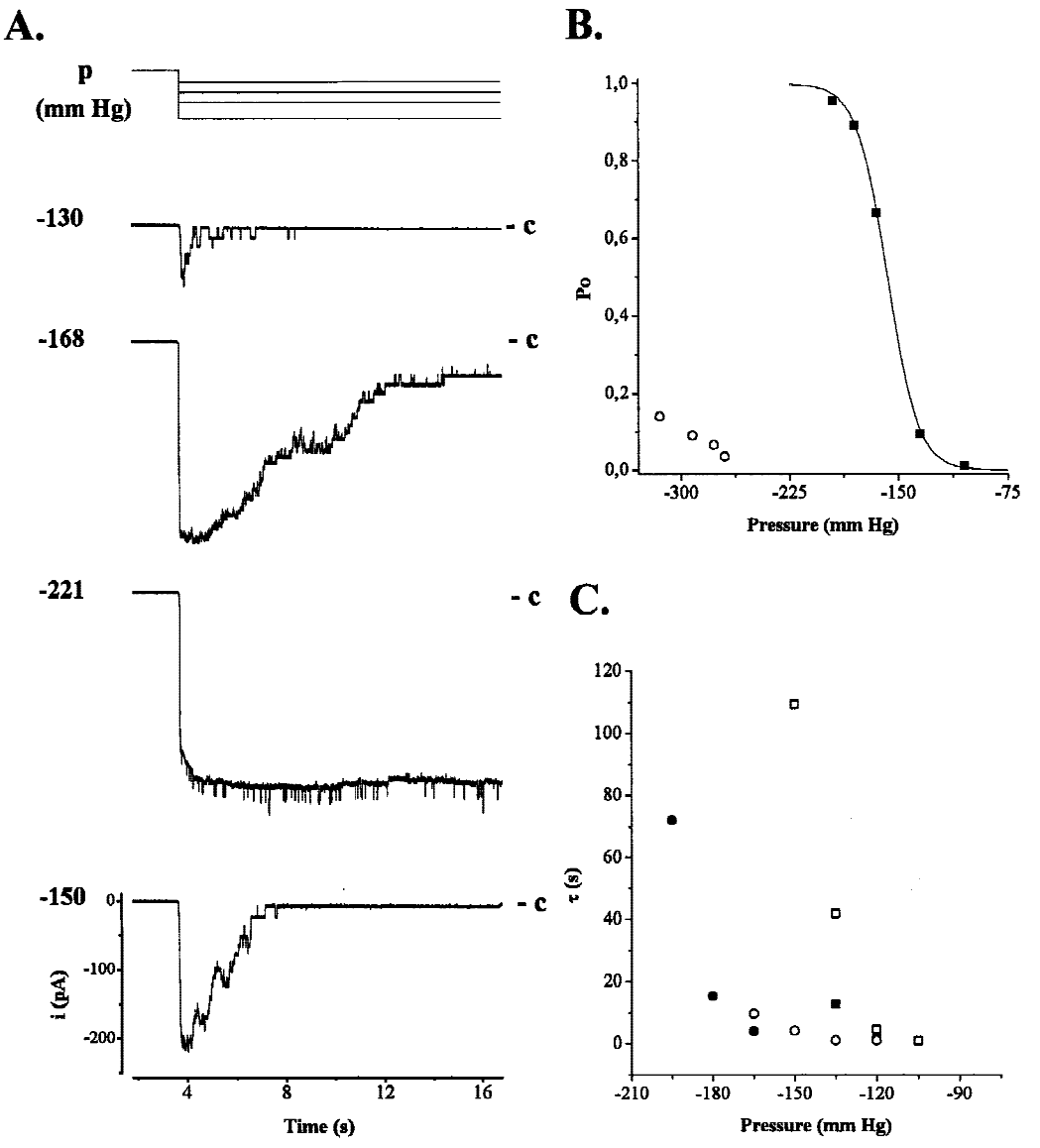

Fig. 1. Properties of 560-pS and 1070-pS MS channels from *E. coli* protoplasts. All traces were recorded and data points were obtained at −15 mV. (*A*) All traces were recorded from the same protoplast patch. Adaptation of the 560-pS MS channel was seen at suctions (negative pressures) 130- and 168-mm Hg (two upper traces). At 221-mm Hg there was a saturation of the 560-pS MS channel current, single openings represent MscL (1070-pS MS channel). The bottom trace was recorded after several applications of strong suction and the adaptation of the 560-pS MS channels remained unchanged. Fitted exponentials had time constants τ of 2, 6.4 and 2.2 sec for 130, 168 and 150-mm Hg, respectively. (*B*) Open probability P_o of the 560-pS (squares) and 1070-pS (circles) MS channels plotted against suction. The data were fitted to the Boltzmann equation (*see* text). (*C*) Adaptation time constant τ of the 560-pS MS channel *vs.* suction. Each data set represents a separate experiment.

distinct. At suctions slightly above their activation thresholds, mean open time of the 560-pS and the 1070-pS MS channels were: 1150 msec (at 130-mm Hg) and 123 msec (at 270-mm Hg) respectively. The dependence of open probability P_o for each MS channel type on suction applied to the recording pipette is shown in Fig. 1*B*. Each data set shows a single, typical experiment. The experimental points were fitted to theoretical curves described by the Boltzmann distribution (shown in Fig. 1*B* for 560-pS MS channel only). The S_p from Boltzmann equation (*see* Materials and Methods) represents the amount of suction that increased P_o by *e*-fold. The average S_p of the 560-pS ion channel was 6.3 ± 2.5-mm Hg ($n = 9$) and the S_p of the 1070-pS channel was 6.5 ± 2.2 mm-Hg ($n = 3$). Thus, the two MS channel types, although showed different kinetics and each of them required different threshold suction for activation, had similar pressure sensitivity in terms of the slope S_p.

In the experiment presented in Fig. 1*A* the 560-pS MS channel showed adaptation and such behavior of this

channel was observed in 117 other excised patches. The adaptation of the MS channel could be also recorded from on-cell protoplast patches (*not shown*). In 3 to 5% of the membrane patches tested we encountered MS channels showing no decline in activity and their kinetics resembled kinetics of the *E. coli* mechanosensitive channel MscS (Martinac et al., 1987; Martinac, Adler & Kung, 1990; Kubalski et al., 1993). The experimental traces of the MS channels showing adaptation could be fitted by single exponentials and the adaptation time constant τ of channel activities shown in Fig. 1*A* was 2.0 sec for 130-mm Hg and 6.4 sec for 168-mm Hg. At saturation (221-mm Hg in this experiment) adaptation might have occurred but was not noticeable within the time range of the suction pulse used (20 sec). The adaptation was not irreversibly lost after strong, saturating stimuli (in Fig. 1*A*, bottom trace was recorded after application of saturating MS current suction of 221-mm Hg), even if membrane patches containing MS channels were exposed to strong suctions repetitively. Such phenomenon was observed in all experiments (over 20 cases), in which this protocol was used. The adaptation time constant τ was dependent on suction applied to the membrane patch. Fig. 1*C* shows the relationship of the time constant τ and suction. Each data set represents a separate patch.

The 560-pS MS channels showed adaptation at positive and negative membrane potentials. Figure 2 shows the experiment in which a fixed suction step was applied to the patch at different membrane voltages. The adaptation time course τ was slightly higher at higher positive and at lower negative voltages. Exponentials fitted to the traces of MS currents recorded at +/−15 mV and +/−30 mV had time constant τ of 3/3.9 sec and of 2.5/2.9 sec respectively. Total open probability NP_o (*see* Materials and Methods) of the MS channels decreased when higher depolarized and lower hyperpolarized potentials were applied. If the NP_o of the MS channels at +15 mV is represented by a value of 1, total open probabilities at +30, +45 and +60 mV were: 1.00 ± 0.34, 0.61 ± 0.16 and 0.45 ± 0.21 respectively (±SD, $n > 3$ in each case). Similarly, the total open probabilities of the MS channels at negative membrane potentials, normalized to the NP_o at −15 mV, were: 0.72 ± 0.14, 0.23 ± 0.13 and 0.19 ± 0.11 at −30, −45 and −60 mV (±SD, $n > 4$ in each case). In this experiment (Fig. 2), at +/−45 mV adaptation of the MS currents was also present but the number of openings was not sufficient to perform proper exponential fit to these recordings. Similar results were obtained in 12 membrane patches. We applied suction pulses at different voltages over 30–60 sec and during this time no sign of recovery to the initial level of MS channel activity was observed.

Our usual experimental procedure was such that suction steps of duration 20 sec were applied 60 sec after

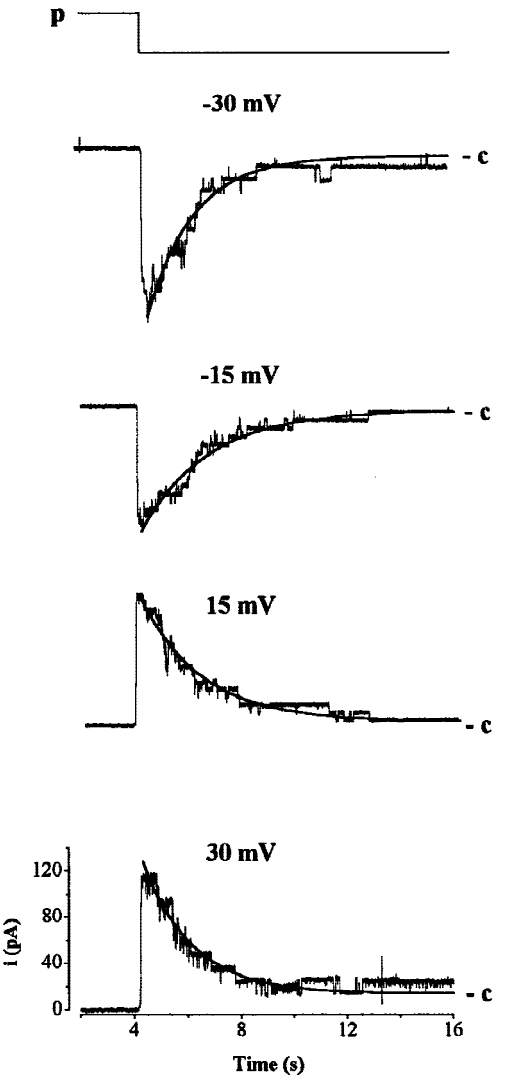

Fig. 2. Adaptation of the 560-pS MS channel was voltage independent. All four traces were recorded from the same protoplast patch. Suction step was 156-mm Hg.

suction of the previous pulse was released. We established this procedure based on results of the experiments in which we investigated the time course of the MS channels recovery from adaptation upon removal of suction. In this set of experiments runs of two pulses of identical strength and duration were applied to membrane patches every 4 min. The suction of the pulses was such that most of the MS channels in the patch were activated and the duration of the pulse was sufficiently long to promote full adaptation of the activated MS channels. The time interval between pulses was different in each run and it was 20, 40, 60 and 120 sec. The open probability NP_o of the MS channels responding to the second pulse was compared to the NP_o during the first pulse represented by a value of 1. The normalized open probabilities NP_o during the second pulse (±SD) were:

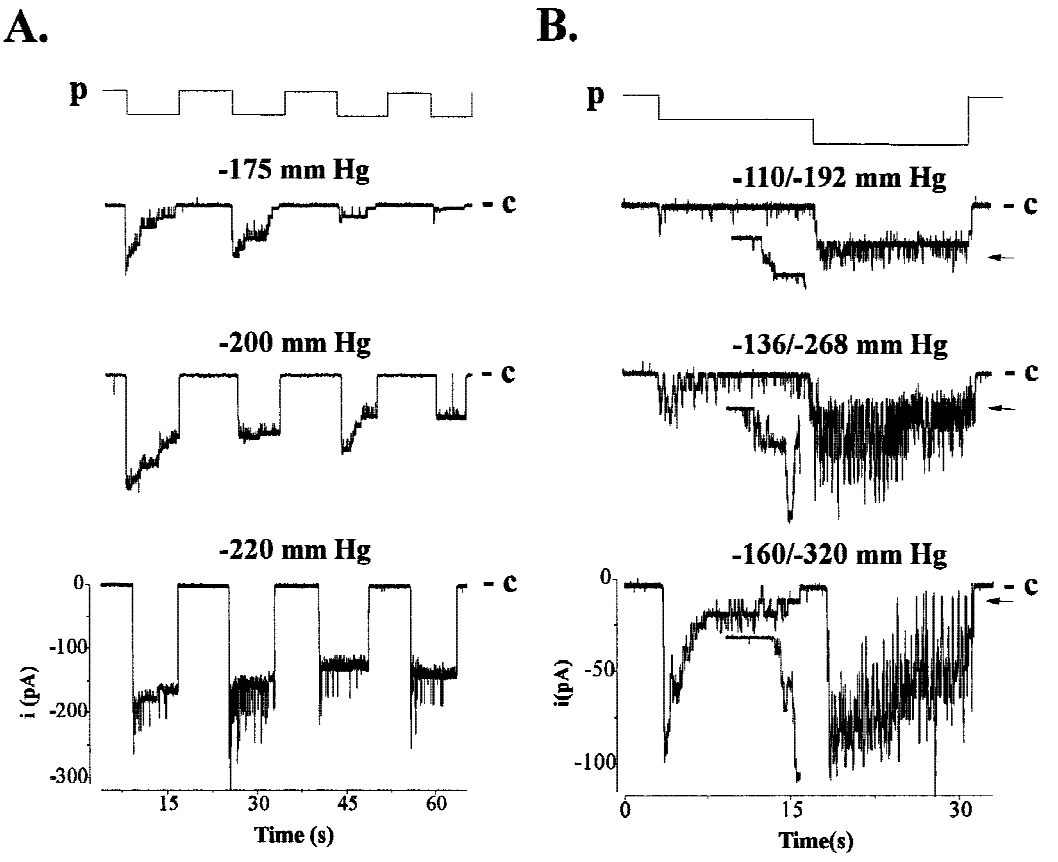

Fig. 3. MS channel response to four- and double-step suction pulses recorded at −15 mV from two different protoplast patches. (*A*) In the four-step protocol, steps of suction were applied every 10 sec for 7–8 sec. Runs of four suction steps were applied 60 sec apart. Adaptation was clearly seen during the first suction step indicating that, in general, the 10-sec interval between steps was too short for the adapted MS channels to recover from adaptation (*see* text for details). (*B*) Three sets of double suction steps were applied 60 sec apart. Inserts (approx. 1-sec long) show the activation slope of the second step. Arrows indicate the highest open level of the 560-pS MS channels during the second step. In the double-step protocol the MS channels adapted to the first step were not reactivated by an increase of suction during the second step. If the first step suction was strong (160-mm Hg), the second suction step activated mostly the 1070-pS MS channels (MscLs).

0.13 ± 0.07 ($n = 4$), 0.55 ± 0.14 ($n = 4$), 0.72 ± 0.15 ($n = 6$) and 0.95 ± 0.31 ($n = 5$) following intervals of 20, 40, 60 and 120 sec, respectively.

In the next set of experiments we investigated whether adaptation is dependent on time and on amount of suction applied. Figure 3*A* shows the experiment in which suction steps (four in a run) were applied every 10 sec for 7–8 sec. All three runs shown in Fig. 3*A* were applied to the same patch and each run of four suction steps was separated from the next one by a period of 60 sec. The suction of the steps was kept constant during each run and in this experiment it was 175-mm Hg in the first run, 200-mm Hg in the second run and 220-mm Hg in the third run. In the first run adaptation of the 560-pS MS channel was present during each suction step, the open probability NP_o, however, was consecutively lower following each application of suction. The open probability NP_o of the MS channel during the fourth suction step was 0.41, comparing to 3.97 during the first suction step of this run. In the second run when 4 steps of 200-mm Hg were applied, the adaptation of the MS channels was clearly distinguished only during the first and the third step and the open probability was also lower following each application of suction. The open probabilities NP_o in the second run were following: 11.64, 7.36, 6.76 and 5.04. In the third run 220-mm Hg was applied and this suction in this patch was sufficient to activate the MscL channels. The adaptation of the 560-pS MS channels was clearly seen only in the first suction step of this run. The total open probabilities of the 560-pS channels in this run were following: 13.33, 12.46, 9.83 and 10.81. Note, that the MscL channels showed an adaptation too. This protocol was applied to the other two patches and similar adaptive behavior was observed. We used also double-step protocol (5 patches) in which a second suction step was applied without releasing the suction of the first step (Fig. 3*B*). In the experiment shown in Fig. 3*B*, three sets of suction steps were applied

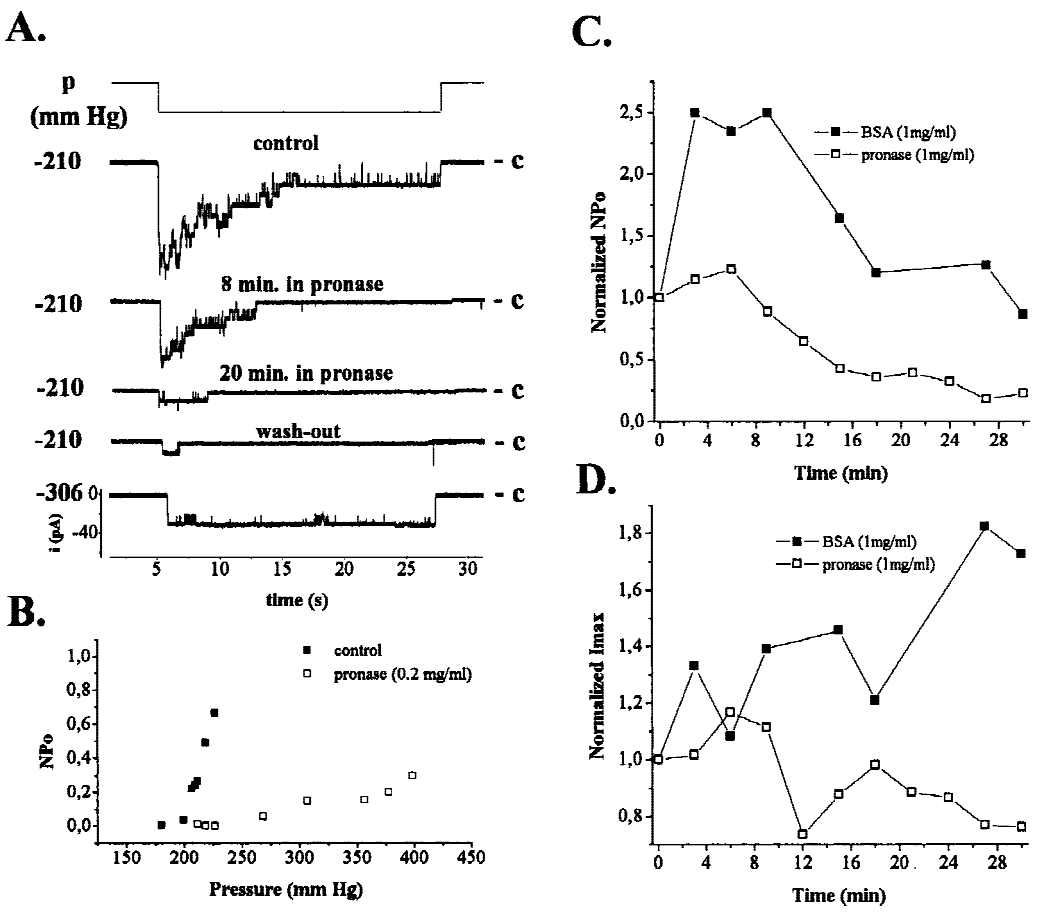

Fig. 4. Effect of proteolysis on the MS channel activity. All traces were recorded and data points were obtained at −15 mV. (*A*) Limited proteolysis (0.2 mg/ml pronase in the bath on the cytoplasmic side of the patch) decreased MS channel activity but did not remove adaptation. Application of strong suction after proteolytic treatment (bottom trace) opened larger number of MS channels but without apparent adaptation. (*B*) The total open probability NP_o data from experiment in (*A*) before and after pronase treatment are plotted *vs.* suction. (*C*) Total open probabilities NP_o in control, during pronase or BSA treatment and after washout were normalized to the control NP_o. The protoplast patches were exposed to pronase (1 mg/ml) or BSA (1 mg/ml) treatment for 27 min, the last data point in each set represents normalized NP_o after washout. Both treatments resulted in an initial increase of NP_o which later decreased to the control value in the case of BSA, and in the case of pronase to 15–20% of its value in the control. (*D*) Normalized (to the control value) maximal number of active MS channels during suction pulse represented by I_{max} from the experiments in (*C*). Pronase treatment decreased the number of active MS channels, whereas more MS channels were activated in the presence of BSA.

to the same membrane patch. Intervals of 60 sec were maintained between sets. The suctions of the first step were 110, 136 and 160-mm Hg and after the MS currents completely adapted, the suctions were increased to approximately twice as high level as that of the first step. The increase of suction to 192-mm Hg in the first set activated five 560-pS MS channels (Fig. 3*B*, upper trace). In the third set (Fig. 3*B*, bottom trace) only one 560-pS MS channel opened in response to increase of suction to 320-mm Hg. Openings of the MscL could be mostly observed during the second step in the second and in the third set (middle and bottom traces). In the double-step protocol, the number of active 560-pS MS channels during the second suction step (marked with arrows in the experiment shown in Fig. 3*B*) was lower following application of stronger suctions during the first step. The 560-pS MS channels did not show apparent adaptation during the second step.

It was postulated in models of mechanotransduction for MS channels from *Xenopus* oocytes (Hamill & McBride, 1992, 1994) and for MS channels from C6 glioma cells (Bowman & Lohr, 1996) that the mechanism responsible for tension transmission can be represented by springs in series with dashpots. The dashpots release tension and would be responsible for channel adaptation. Bowman and Lohr (1996) proposed that this viscoelastic element is a membrane-associated cytoplasmic domain of the MS channel. To investigate whether such an element is present in the *E. coli* 560-pS MS channel, we exposed the cytoplasmic side of the membrane patch to

proteolytic digestion. We applied pronase at concentrations of 0.2 mg/ml and/or 1 mg/ml to 12 membrane patches containing MS channels and Fig. 4 summarizes our experience from these experiments; 25 to 45 min after the experimental chamber was perfused with bath solution containing 0.2 mg/ml pronase the open probability of the MS channel decreased (Fig. 4*A* and *B*). Suctions that resulted in saturation of the MS current in untreated membrane patches, opened only one or two MS channels after pronase digestion. In the experiment shown in Fig. 4*A*, 210-mm Hg opened 14 MS channels in control and 1 MS channel after pronase treatment. These channels still showed adaptation. Higher suctions resulted in opening of larger number of channels but adaptive behavior could not be observed. Figure 4*B* shows total open probability NP_o of the MS channels from the experiment shown in Fig. 4*A* plotted against applied suction before and after pronase treatment. Application of 1 mg/ml pronase abolished MS channels activities entirely. After exposure of membrane patches (7 cases) for 25–30 min to 1 mg/ml pronase and after washout, MS channel activity could not be seen even at very high suctions, causing sometimes patch breakage. Two membrane patches were first exposed to 0.2 mg/ml and then to 1-mg/ml pronase and the latter concentration abolished the MS channel activity that remained after the treatment at the former concentration. When *E. coli* protoplast membrane patches were subjected to pronase digestion (at both concentrations) over the first five minutes an increase in NP_o was observed. To investigate this phenomenon and to perform a control experiment for pronase digesting activity we studied an effect of bovine serum albumin (BSA) on the 560-pS MS channel (4 cases). Figure 4*C* shows normalized total open probabilities (NP_o) of the 560-pS MS channels from two different membrane patches, exposed either to pronase or BSA at concentration 1 mg/ml, plotted against time. A value of 1 for normalized NP_o represents MS channel activity that is as high as that of untreated channels. Suction pulse was constant throughout each experiment. In both cases, over the first minutes after application of pronase or BSA, we observed an increase in the MS channel NP_o. After 27 min, exposure to BSA and washout, the NP_o of the MS channels returned to its control value. In the case of pronase, an initial increase of activity was followed by a decrease of the MS channel activity to a level lower than in the control, apparently as a result of pronase digestion of cytoplasmic domains of the MS channels. In Fig. 4*D* we plotted normalized peak MS current I_{max} (which corresponds to the maximal number of open channels) against time for the two experiments from Fig. 4*C*. A value of 1 represents control I_{max} in each experiment. We observed a reduced number of active channels after exposure of the membrane patch to pronase, whereas a similar concentration of BSA resulted in an increase of the number of active MS channels.

The adaptive behavior of the bullfrog sacculus hair cells was Ca^{2+} dependent (Eatock, Corey & Hudspeth, 1987; Assad, Hacohen & Corey, 1989). We perfused our experimental chamber with a bath solution containing 10^{-6} M Ca^{2+} and with an increased concentration of Mg^{2+} substituting Ca^{2+} (3 cases). We did not observe any change in MS channel adaptation in these experiments when the inner leaflet of the membrane patch was exposed to low Ca^{2+} (*not shown*). We did not apply low concentration of Ca^{2+} to the outer leaflet of the membrane patch.

Discussion

The results in this study present some characteristics of the adaptive behavior of the 560-pS MS channels from *E. coli* protoplasts. We think that the adaptation represents an intrinsic property of the *E. coli* MS channels and is not associated with change of geometry of the membrane patch caused by its movement within the glass capillary. As it has been demonstrated (Sokabe & Sachs, 1990; Bowman & Lohr, 1996), membrane patch moves away or toward the tip of the glass capillary following application of negative or positive pressure respectively. The time course of the adaptation of the *E. coli* MS channels is much slower comparing to time courses of adaptation seen in hair cells, *Xenopus* oocytes and glioma cells (Hamill & McBride, 1992; Bowman & Lohr, 1996). Therefore, it can be argued that the adaptation of *E. coli* MS channels observed in our experiments during sustained pressure may be associated with a membrane patch ability for relaxation and its possible movement toward its original position within the glass pipette. This movement may result in a decrease of tension in the membrane patch and would cause closures of the MS channels. Work by Bowman and Lohr (1996) proved, however, that membrane patches from C6 glioma cells did not return to their original positions 10 sec after applied suction had been released, although their planar geometry was restored. Therefore we expect that in our four-pulse experiment, when pulses were applied in 10-sec intervals, the membrane patch was pulled by the first suction step to a different position and remained in this position arrested as an almost static patch. As we have demonstrated, the MS channels under these conditions retained their mechanosensitivity and ability for adaptation.

There is a significant difference between experiments performed on membrane patches from *E. coli* or yeast spheroplasts, *Xenopus* oocytes or glioma cells and our experiments. *E. coli* protoplasts should contain only the cytoplasmic (inner) membrane and not the outer membrane. The outer, and not the inner membrane, is

firmly attached to the sacculus (cell wall) by the covalently linked lipoproteins and by the outer membrane porins: the *omp*C and *omp*F gene products (Park, 1996). Therefore, the membrane restraining element, cell wall, should not be present in our preparation. This fact and/or properties of the channel molecule itself may be responsible for the observed MS channel-adapting behavior. There are two features, which distinguish *E. coli* MS channel-adapting behavior from adaptation of other MS channels. This behavior was not fragile and, consistently in all experiments, repeated application of strong (saturating MS current) suctions remained without any effect on the adaptation and its time course. Another distinguishing feature of the *E. coli* MS channel adaptation is its weak voltage dependence. The time course of adaptation was only slightly different at positive and negative voltages and this was observed in all membrane patches tested. These two features may reflect a physiological importance of the adaptive behavior of the MS channel which is not reduced or lost after application of very high suctions and voltage of any polarity.

In our four-pulse experiment (Fig. 3) we were losing a portion of active channels each time a suction pulse was applied. In general, the adapted MS channels did not open when the next suction step was applied because the time necessary for recovery from adaptation was too short (*see* steps 4–3 at 175-mm Hg; 2–1, 4–3 at 200-mm Hg; all four steps at 220-mm Hg). However, when four 175-mm-Hg pulses were applied, adapted MS channels could reopen during the following suction step (*see* steps 1, 2, 3) after recovery from adaptation. In this case, we can assume, at lower suctions (in this experiment such phenomenon was observed at suctions 150 (*not shown*) and 175-mm Hg) there is a population of MS channels that recover more easily from an adapted state. At higher suctions (220-mm Hg in the experiment shown in Fig. 3*A*) adaptation was present in the first two steps but at a much slower time course because, as we have shown in Fig. 1*C*, adaptation time constant τ was dependent on suction. The MS channels that adapted in the two first steps of this run did not reopen in the third and in the fourth step. The double-step protocol (Fig. 3*B*) expanded our findings from the four-pulse experiment in that the recovery from an adapted state required the applied suction to be released. Most of the MS channels adapted to 160-mm Hg did not reopen when sustained suction pulse was increased to 320-mm Hg (Fig. 3*B*, bottom trace). Lower suctions of the first step (110 and 136-mm Hg, upper and middle traces of Fig. 3*B*) resulted in lower number of active channels during this step and therefore in a lower number of adapted channels in the patch. Although we cannot discriminate between channels which were active during the first step and those that were active during the second step, we think that channels which were open during the second step were those that were not active during the first step. The 560-pS MS channel opened during the second step did not show apparent adaptive behavior due to a large adaptation time constant τ. A similar protocol was used for the MS channels from *Xenopus* oocytes (Hamill & McBride, 1992). In this study, the suction of the second pulse was twice as high as the suction of the first one and the response of the adapted MS channels to the second pulse was similar in amplitude and adaptation course to the response to the first pulse. The authors concluded that, since the adapted MS channels could be reactivated instantaneously after adaptation and showed a similar response to the second suction step, the channels were not inactivated but their gating sensitivity had changed as a result of application of the first suction step. Although in the range of very low suctions we also can see activation of the MS channels during the second step the adapted *E. coli* MS channels required time and release of the applied suction for their recovery.

In the next set of experiments we applied pronase to the cytoplasmic side of the membrane patches. Exposure of membrane patches to pronase or BSA resulted in an initial increase of open probability NP_o of the MS channels along with an increase of the number of active channels (increase of peak current I_{max}) (Fig. 4*C* and *D*). We do not know how to interpret this phenomenon and further studies are required to clarify it. After 10–15 min the MS channels exposed to pronase showed a decline in both NP_o and I_{max} presumably as a result of pronase digestive activity. These experiments proved that the *E. coli* 560-pS MS channel shows adaptation as long as it senses tension in the membrane. Application of 0.2-mg/ml pronase reduced NP_o of the MS channels without removing adaptation (Fig. 4*A*). After pronase treatment the adaptation was still present if one or two MS channels opened and NP_o was < 0.02; at higher suctions and higher open probabilities, the time constant τ was high and the adaptation was not observed. Application of 1 mg/ml of pronase removed responsiveness of the channels to suction, and we assume, the tension transmission mechanism responsible for opening of the MS channels was disrupted. A lower number of open channels after limited proteolysis (Fig. 4*D*) also suggest that the MS channels with removed fragments did not sense membrane tension and did not open, even if higher suctions were applied. These results may suggest that a common domain of the MS channel molecule may be responsible for both tension sensing and adaptation. Our results do not discriminate, however, between the possibility that the tension sensing and adaptation mechanisms involve separate domains and both, or the tension-sensing domain only, are sensitive to pronase digestion. This domain (or domains) is located on the cytoplasmic side of the inner membrane. It has been recently demonstrated that mutants with N-terminal deletions or changes to the N-terminal amino acid sequence in the *E. coli* MscL exhibited altered pressure sensitivity and gating and,

thus, the N-terminus may represent a "mechanosensitive structural element" of this MS channel (Häse et al., 1997). The 560-pS MS channel may have similar tension sensing mechanism as MscL since both MS channels show very similar sensitivity to suction.

Our results may also implicate a possible mechanism of introducing mechanosensitivity into the gating of the MS channel molecule. There are two general models of tension transmission to the MS channel (for recent review, *see* Hamill & McBride, 1997): one relies on the extracellular matrix and/or cytoskeleton (tethered model), and in the other, the tension is transmitted via lipid bilayer (bilayer model). Since the protoplast membrane should be devoid of the cell wall, the MS channel could be activated by the bilayer mechanism. The experiments with pronase in which removal of cytoplasmic segments of the MS channel led to loss of mechanosensitivity suggest, however, that the mechanism which might be a combination of the two models may be responsible for tension transmission to the *E. coli* MS channel. In this model tension is derived within the lipid bilayer, but it would be exclusively sensed by MS channel cytoplasmic regulatory domain anchored in the lipid bilayer.

In summary, the adaptive behavior of the *E. coli* MS channel was voltage independent and was not irreversibly lost after strong, saturating MS current suctions. The adaptation could not be removed by pronase digestion, since, as long as the MS channel responded to suction, it also showed adaptation. The *E. coli* 560-pS MS channel needed more time to adapt than reported for other MS channels (adaptation time constant τ, found in this study, was in the range of seconds compared to milliseconds). It also needed time to restore its sensitivity to the suction. Loss of sensitivity to stretch of the 560-pS MS channel after adaptation may have deleterious consequences to *E. coli*. However, as it has been demonstrated in the double-pulse protocol (Fig. 3*B*), after the 560-pS MS channels had adapted, the *E. coli* membrane still had the capacity to respond to stretch by opening of the MscLs. The presence of MscL in the *E. coli* inner membrane might be of vital importance for this bacterium.

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